Supplemental Information

Target species

Oreochromis mossambicus (Mozambique tilapia) and *Tilapia mariae* (spotted tilapia) are native to Africa yet both were introduced to Queensland Australia in the 1970s through the aquarium trade and have since spread (Kroon, et al., 2015). *Tilapia mariae* are more restricted in their invasion with established populations found only in catchments between Innisfail and Cooktown in Australia, whereas *O. mossambicus* are more widespread throughout Australia with confirmed sightings in Queensland (approx. 20 of 76 catchments), Northern Rivers region in New South Wales, and Pilbara region in Western Australia (Webb, 2007; Bradford, et al., 2011; Russell, et al., 2012).

Rhinella marina (cane toad) is native to Latin America and was introduced to north-eastern Australia in 1935 in an attempt to biologically control harmful sugarcane pest insects (Turvey, 2013; Tingley, et al., 2017) but has since spread across >1.2 million km^2 of tropical and subtropical Australia (Urban, et al., 2008). and has had devastating impacts on native biodiversity, especially large anuran-eating predators (e.g., marsupial quolls, freshwater crocodiles, varanid and scincid lizards, and elapid snakes) due to their lethal endogenous toxin (Lentic, et al., 2008; Doody, et al., 2009; Shine, 2010; Jolly, et al., 2015; Fukuda, et al., 2016).

Cabomba caroliniana (fanwort) is a submerged aquatic perennial plant that is native to North and South America (Ørgaard, 1991) that was first found naturalized in Australia in 1967 (Wilson, et al., 2007) and is now classified as a "weed of national significance" (Day, et al., 2014). In late summer, *C. caroliniana* stems become brittle and break apart, which facilitates distribution and invasion into new water bodies. The main methods of invasive spreading are vegetative (e.g., seed broadcasting) and fragmentation (e.g., $\geq$ 1 cm floating stem fragments or leaves) as leaf nodes can take root and grow into new plants (Schooler and Julien, 2006; Bickel, 2012; McCracken, et al., 2013; Bickel, 2015; Bickel, 2017); however, viable reproduction from seed has been reported in the Top End of the Australian Northern Territory (Schooler, et al., 2009).

Assay Design

The presence of multiple mitochondria or chloroplast within each cell makes mitochondrial or chloroplast DNA more abundant and thus more detectable than nuclear DNA within environmental water samples (Goldberg, et al., 2016; Braukmann, et al., 2017; Kuzmina, et al., 2017). Moreover, mitochondrial *16S* and chloroplast *matK* genes are commonly targeted by barcoding studies and thus nucleotide sequence information from a broad range of species is available within the National Center for Biotechnology Information public database (GenBank; www.ncbi.nlm.nih.gov).

Initial tilapia assay (16S Oreo-F and 16S Tilapia-R; Robson, *et al*., 2016) contains 0 and 3 mismatches with *O. mossambicus* and *T. mariae 16S* nucleotide sequences, respectively, whereas revised tilapia assay (Table 4) has 0 mismatches to both invasive tilapia species (Edmunds and Burrows, 2019a).

Tingley, et al (2019) successfully detected cane toads in filtered environmental water samples using a TaqMan-based assay that targets a short 80 bp fragment spanning the tRNA-Gly and NADH dehydrogenase subunit 3 (ND3) regions of mitochondrial genome; however, we had already developed an alternative Sybr-based *R. marina* assay that targets a larger 290 bp fragment of *16S* mitochondrial gene (R.marina_16S; Edmunds and Burrows, 2019b).

Scriver, et al., (2015) developed a *C. caroliniana* eDNA assay that targeted maturase K (*matK*) gene within chloroplast genome but it failed *in vitro* validation; therefore, we developed a new *matK* assay for *C. caroliniana* eDNA detection within Australian waterways (C.caroliniana_matK; Edmunds and Burrows, 2019c).

Geneious analysis software (version R11; Kearse et al., 2012) was used to obtain all available sequences for *O. mossambicus* and *T. mariae 16S* (n = 11), *R. marina 16S* (n = 27), and *C. caroliniana matK* (n = 13). Sequences for each species were aligned using ClustalW (Thompson, et al., 2003) and assessed for conserved regions wherein target species exhibited ≥ 1 base pair mismatch with humans and non-target Australian fishes (n = 82), frogs (n = 18), turtles (n = 12), and aquatic plants (n = 36) (Supplemental Tables 2 and 3).

Quantitative PCR

In vitro validations 3 - 7 (Figure 1; Supplemental Figures 1 – 5) [see Preservatives and terminal precipitants; Longmire's short-term preservation effectiveness; Lysis duration and ±PCI purification; NaCl, ±Glycogen, and ±PCI purification (high-copy clean-spike); and Lysis duration and glycogen concentration] used initial tilapia assay (Robson, et al., 2016) and were run as 20 µL reactions that contained: (1) 10 µL QuantiFast® qPCR master mix (Qiagen Australia Pty Ltd), (2) 1 µL each primer (10 µM stock), (3) 2 µL MilliQ® water (Thermo Fisher Scientific Australia Pty Ltd), and (4) 6 µL template. Thermal cycling conditions were: (1) initial denaturation at 95˚C for 10 min, (2) 40 cycles of 95˚C for 10 secs and 60˚C for 30 sec, and (3) terminal dissociation curve generation (60˚C to 95˚C at 0.15˚C/sec). QuantStudio™ Design and Analysis Software (version 1.4.2; Thermo Fisher Scientific Australia Pty Ltd) was used to set the threshold fluorescence to 0.1 before data was exported in Microsoft Excel format.

All other *in vitro* and *in situ* validations (Figures 3 – 5; Supplemental Figures 6 - 8) [see Assay efficiency; Assay specificity; Centrifugation, glycogen, and inhibitor purification (low-copy clean-spike); Glycogen and inhibitor purification (low-copy tank-spike); Double inhibitor purification (silica versus silica-Zymo); Longmire's long-term preservation effectiveness; and *In situ* validations] used Tilapia_v2_16S, R.marina_16S, or C.caroliniana_matK assays (Table 4) and were run as 10 µL reactions that contained: (1) 5µL PowerUp® qPCR Mastermix (Thermo Fisher Scientific Australia Pty Ltd), (2) 0.5, 0.25, or 0.9µL each primer (10µM stock for 500, 250, or 900 nM final), (3) 1, 1.5, or 0.2µL MilliQ® water (Thermo Fisher Scientific Australia Pty Ltd), and (4) 3 µL template, respectively. Thermal cycling conditions were: (1) initial UDG incubation at 50˚C for 2 min, (2) initial denaturation at 95˚C for 2 min, (3) 45 cycles of 95˚C for 15 secs then 60˚C (initial and Tilapia_v2_16S) or 65˚C (R.marina_16S and C.caroliniana_matK) for 1 min, and (4) terminal dissociation curve generation (60˚C to 95˚C at 0.15˚C/sec). QuantStudio™ Design and Analysis Software (Thermo Fisher Scientific Australia Pty Ltd) was used to set the threshold fluorescence to 0.2 for all species-specific assays before data was exported in Microsoft Excel format.

***In silico* validation**

Initial targeted PrimerBLAST specified a list of Australian freshwater fish, frogs, turtles, and aquatic plants (Supplemental Table 1) against which each assay was assessed for potential primer binding. For this targeted search, all species with ≤ 5 base pair mismatches to Tilapia_v2_16S, R.marina_16S, and C.caroliniana_matK assays were recorded, respectively.

Subsequent *in silico* test used non-targeted PrimerBLAST (i.e., no species specified) to test each species-specific assay against all species with nucleotide sequences deposited in NCBI "nr" database to ascertain which, if any, species might be amplified. For this non-targeted search, all species with ≤ 2 base pair mismatches to Tilapia_v2_16S, R.marina_16S, and C.caroliniana_matK assays were recorded, respectively.

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